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Frank C. Eisenschenk, Ph.D., Patent Attorney

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 CFR 1.322
Docket No. UF.T398XC1
Patent No. 7,510,852

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Monique Royer, Dean W. Gabriel, Roger Frutos, Philippe Rott
Issued	:	March 31, 2009
Patent No.	:	7,510,852
Conf. No.	:	6228
For	:	Biosynthetic Genes and Host Cells for the Synthesis of Polyketide Antibiotics and Method of Use

Mail Stop Certificate of Corrections Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION
UNDER 37 CFR 1.322 (OFFICE MISTAKE)

Sir:

A Certificate of Correction for the above-identified patent has been prepared and is attached hereto.

In the left-hand column below is the column and line number where errors occurred in the patent. In the right-hand column is the page and line number in the application where the correct information appears.

Patent Reads:	**Application Reads:**
Column 4, line 23:	Amendment Under 37 CFR § 1.111 dated June 26, 2008, page 2, paragraph [0015]:
"P14687 SEQ ID NO: 132"	--P14687 (SEQ ID NO: 132)--

Column 4, line 56:	Page 5, paragraph [0022], line 2:
"RitE-1"	--RifE-1--
Column 10, line 60:	Page 12, paragraph [0047], line 4:
"Vols. I, II, and II"	--Vols. I, II, and III--
Column 16, line 19:	Page 19, paragraph [0073], line 5:
"Proc. Natl. Acad. Sci. U.S."	--Proc. Natl. Acad. Sci. U.S.A.--
Column 16, line 23:	Page 19, paragraph [0073], line 6:
"Proc. Natl. Acad. Sci. U.S."	--Proc. Natl. Acad. Sci. U.S.A.--
Column 22, line 21:	Page 26, paragraph [0094], lines 6-7:
"protecting a plant; against"	--protecting a plant against--
Column 22, line 40:	Page 26, paragraph [0096], line 6:
"kanamnycin"	--kanamycin--
Column 26, line 31:	Page 31, paragraph [00109], line 9:
"(5'tgeccacaggccgtcgagt3')"	--(5'tgcccacaggccgtcgagt3')--
Column 27, line 4:	Page 31, paragraph [00110], line 11:
"SEQ D No: 6"	--SEQ ID No: 6--
Column 27, line 51:	Page 32, paragraph [00112], line 10:
"J-ketoacyl synthase"	--β-ketoacyl synthase--
Column 29, line 22:	Page 34, paragraph [00118], line 1:
"albXV"	--albXI--
Column 29, line 60:	Page 35, paragraph [00120], line 7:
"motifl involved"	--motif I involved--

Column 31, line 15:	Page 36, paragraph [00123], line 2:
"AlbXVI"	--AlbXVII--
Column 31, line 20:	Page 36, paragraph [00124], line 1:
"albXVII"	--albXVIII--
Column 31, line 21:	Page 36, paragraph [00124], line 2:
"AlbXIII"	--AlbXVIII--
Column 31, lines 35-36:	Page 36, paragraph [00124], line 11:
"(5'cgttgaggatgcagcgctcg31')"	--(5'cgttgaggatgcagcgctcg3')--
Column 34, line 7:	Page 39, paragraph [00136], line 11:
"from *Comamnonas*"	--from *Comamonas*--
Column 36, line 9:	Page 42, paragraph [00140], line 6:
"BLm (&Ala)"	--Blm (β-Ala)--
Column 36, line 27:	Page 42, paragraph [00141], line 7:
"confined"	--confirmed--
Column 36, line 58:	Page 43, paragraph [00143], line 1:
"from albX"	--from albXIX--
Column 40, lines 63-64:	Page 47, paragraph [00155], lines 13-14:
"and XhoSalXaHT1PGF 5'cgatatcgccttcctcgagggtategataagc3'"	--and XhoSalXaHTPGF 5'cgatatcgccttcctcgagggtatcgataagc3'--
Column 41, line 14:	Page 48, paragraph [00156], lines 1-2:
"pUFR043-pOp34/XALB2-3"	--pUFR043-pOp3-4/XALB2-3--

Column 41, line 15:	Page 48, paragraph [00156], line 2:
"pAlb571-pOp3A4XALB2-3"	--pAlb571-pOp3-4/XALB2-3--
Column 42, line 29:	Page 49, paragraph [00160], lines 4-5:
"Because albXVII"	--Because albXVIII--
Column 42, line 46:	Page 49, paragraph [00161], line 6:
"confining"	--confirming--
Column 43, line 51:	Page 51, paragraph [00165], line 7:
"stain Xa13"	--strain Xa13--
Column 43, line 64:	Page 51, paragraph [00166], line 3:
"and albIV)"	--and albIX)--
Column 45, line 47:	Page 53, paragraph [00169], line 9:
"PKS4 module"	--PKS-4 module--
Column 47, line 58:	Page 56, paragraph [00174], line 12:
"AbVII HBCL"	--AlbVII HBCL--
Column 49, line 14:	Page 57, paragraph [00179], line 4:
"identical to albX)"	--identical to albXXI)--
Column 51, Table 1, row "pBC/f":	Page 60, Table 1, row "pBC/F":
"2.5 kb Kpn I-EdoR I"	--2.5 kb Kpn I-EcoR I--
Column 53, Table 1, row "pBKS/XALB3XhoI":	Page 61, Table 1, row "pBKS/XALB3XhoI":
"pBKS/XALB3 with a KhoI site"	--pBKS/XALB3 with a XhoI site--

Column 60, Table 4, row "AlbXIII", Columns "Identities", "Positives", "Gaps":	Page 66, Table 4, "row AlbXIII", Columns "Identities", "Positives", Gaps":
"43/156 56/156 44/156 (28%)"	--43/156 56/156 44/156 (28%)--
Column 66, Table 10, row "Tetracycline":	Page 72, Table 10, row "Tetracycline":
"DH5αKT DHSαAlb′KT"	--DH5αKT DH5αAlb′KT--
Column 264, line 26:	Amendment Under 37 CFR § 1.111 dated June 26, 2008, original claim 65 (renumbered as claim 4):
"36, 37, 39, 39, 40"	--36, 37, 38, 39, 40--
Column 264, lines 30-31:	Amendment Under 37 CFR § 1.111 dated June 26, 2008, original claim 66 (renumbered as claim 5):
"36, 37, 39, 39, 40"	--36, 37, 38, 39, 40--.

A true and correct copy of pages 5, 12, 19, 26, 31, 32, 34, 35, 36, 39, 42, 43, 47, 48, 49, 51, 53, 56, 57, 60, 61, 66, and 72 of the specification as filed and a copy of the Amendment Under 37 CFR § 1.111 dated June 26, 2008 which support Applicants' assertion of the errors on the part of the Patent Office accompanies this Certificate of Correction.

Applicants respectfully assert that the Examiner's amendment to original claims 65 and 66 that accompanied the Notice of Allowance did not amend the succession of SEQ ID NOs contained therein and that the error in both claims was introduced when the claims were retyped.

Approval of the Certificate of Correction is respectfully requested.

Respectfully submitted,

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FCE/jb/sl

Attachments: Copy of pages 5, 12, 19, 26, 31, 32, 34, 35, 36, 39, 42, 43, 47, 48, 49, 51, 53, 56, 57, 60, 61, 66, and 72 of the specification
Copy of Amendment Under 37 CFR § 1.111 dated June 26, 2008
Certificate of Correction

[0018] Figure 7B shows sequences identified as a putative unidirectional promoter upstream from *albXIX* for transcriptional control of operon 5 if *albXVIII* is not expressed.

[0019] Figure 8 is a physical map and genetic organization of the DNA region containing the gene clusters XALB2 and XALB3 involved in albicidin production.

[0020] Figure 9A is linear model 1 leading to the biosynthesis of only one polyketide-polypeptide albicidin backbone.

[0021] Figure 9B is linear model 2 leading to the biosynthesis of four different polyketide-polypeptide backbone.

[0022] Figure 10A is an alignment of the conserved motifs in AT domains from RifA-1, -2, -3, RifB-1, RifE-1 (Rifamycin PKSs, August *et al.*, 1998) and BlmVIII (Bleomycin PKS; Du *et al.*, 2000).

[0023] Figure 10B is a comparison of AlbXIII, FenF (a malonyl-CoA transacylase located upstream from *mycA*, Duitman *et al.*, 1999) and LipA (a lipase; Valdez *et al.*, 1999).

[0024] Figure 11A is a proposed model for biosynthesis of albicidin, including putative substrates of PKS and NRPS modules.

[0025] Figure 11B shows the proposed compositions and structures of albicidins.

[0026] Figure 12 illustrates subcloning of operons 3 and 4 (from pALB540), XALB2 (from pAC389.1) and XALB3 (from pEV639) into a single plasmid, pOp3-4/XALB2-3. A *Bam*HI–*Pst*I fragment from pALB540, corresponding to a portion of operon 4, was subcloned into pBCKS(+), yielding pBC/Op4D (step 1). A *Xho*I site was introduced into this vector immediately upstream from the *Bfr*I site by directed mutagenesis, yielding pBC/Op4DXhoI (step 2). The *Eco*RI fragment from pAC389.1 (XALB2) was then subcloned into pBC/Op4DXhoI, yielding pBC/Op4D/XALB2 (step 3). A *Bfr*I fragment from pALB540 containing complete operon 3 and the beginning of operon 4 was subcloned into pBC/Op4D/XALB2, yielding pBC/Op3-4/XALB2 (step 4). The *Sal*I fragment from pEV639 (XALB3) was subcloned into pBKS, yielding pBKS/XALB3 (step 5). The *Sal*I site located on the *Kpn*I side of the polylinker was then destroyed and substituted by a *Xho*I restriction site, yielding pBKS/XALB3XhoI (step 6). Finally, the *Xho*I cassette of pBC/Op3-4/XALB2 was subcloned into the *Sal*I restriction site of pBKS/XALB3XhoI, yielding pBKS/Op3-4/XALB2-3 (step 7). An *Xho*I site was added to the BamHI site of pLAFR3, yielding pLAFR3XhoI (step8). The *Xho*I cassette from pBKS/Op3-4/XALB2-3 was then cloned into pLAFR3XhoI, yielding pOp3-4/XALB2-3 (step 9).

DETAILED DESCRIPTION OF THE INVENTION

[0027] The invention results from the DNA sequencing of the complete major gene cluster XALB1, as well as the noncontiguous fragments XALB2 and XALB3. XALB1 is present in the two overlapping DNA inserts of clones pALB540 and pALB571. Reading frame analysis

LASER beam-induced perforation of cell wall, or simply by incubation with or without polyethylene glycol (PEG). Plants transformed with a genetic construct of the invention may be produced by standard techniques known in the art for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transferability. Agrobacterium transformation is used by those skilled in the art to transform algae and dicotyledonous species. Substantial progress has been made towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants. In particular, Agrobacterium mediated transformation has now emerged as a highly efficient transformation method in monocots. Microprojectile bombardment, electroporation, and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g., bombardment with Agrobacterium-coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

[0047] Following transformation, a plant may be regenerated, e.g., from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues, and organs of the plant. Available techniques are reviewed in Vasil et al. (1984) in Cell Culture and Somatic Cell Genetics of Plants, Vols. I, II, and III, Laboratory Procedures and Their Applications (Academic press); and Weissbach et al. (1989) Methods for Plant Mol. Biol.

[0048] The transformed plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited, and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved.

[0049] The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practicing the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

[0050] Also according to the invention, there is provided a plant cell having the constructs of the invention. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector including the construct into a plant cell. For integration of the construct into the plant genome, such introduction will be followed by recombination between the vector and the plant cell genome to introduce the sequence of

terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes simplex thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic vectors containing promoters such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., 1983, Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, and/or the alkaline phosphatase promoter.

[0074] The vectors according to the invention are, for example, vectors of plasmid or viral origin. In a specific embodiment, a vector is used that comprises a promoter operably linked to a nucleic acid sequence encoding a polypeptide as disclosed herein, one or more origins of replication, and, optionally, one or more selectable markers (e.g., an antibiotic resistance gene). Expression vectors comprise regulatory sequences that control gene expression, including gene expression in a desired host cell. Exemplary vectors for the expression of the polypeptides of the invention include the pET-type plasmid vectors (Promega) or pBAD plasmid vectors (Invitrogen) or those provided in the examples below. Furthermore, the vectors according to the invention are useful for transforming host cells so as to clone or express the polynucleotide sequences of the invention.

[0075] The invention also encompasses the host cells transformed by a vector according to the invention. These cells may be obtained by introducing into host cells a nucleotide sequence inserted into a vector as defined above, and then culturing the said cells under conditions allowing the replication and/or the expression of the polynucleotide sequences of the subject invention.

[0076] The host cell may be chosen from eukaryotic or prokaryotic systems, such as for example bacterial cells, (Gram negative or Gram positive), yeast cells (for example, Saccharomyces cerevisiae or Pichia pastoris), animal cells (such as Chinese hamster ovary (CHO) cells), plant cells (e.g., algae), and/or insect cells using baculovirus vectors. In some embodiments, the host cells for expression of the polypeptides include, and are not limited to, those taught in U.S. Patent Nos. 6,319,691, 6,277,375, 5,643,570, or 5,565,335, each of which is incorporated by reference in its entirety, including all references cited within each respective patent.

[0077] Furthermore, a host cell strain may be chosen which modulates the expression of

by a process that includes at least three proteins coded by the nucleic acids of the subject invention in combination with additional enzymes that modify the product to provide a non-naturally occurring Albicidin-like product having at least one of the useful properties reported for albicidin. In certain embodiments, the antibiotic or antibiotics have at least one of the general structures illustrated in Figure 11. In other embodiments, antibiotics of the subject invention have at least 4 of the structural elements illustrated in Figure 11, and an elemental composition of $C_{40}H_{35}N_6O_{15}$.

[0094] The invention further provides a method of protecting a plant against damage from albicidin that comprises applying an agent that blocks expression at least one gene in the Albicidin Biosynthetic Gene Clusters to the plant to be protected. Additional inventions include a method of obtaining agents useful in blocking expression of albicidin by screening materials against a modified host cell line that expresses the Albicidin Biosynthesis Gene Clusters and selecting for materials that stop or decrease albicidin production and a method of protecting a plant against phytotoxic damage from an antibiotic that comprises inserting into the plant and operably expressing at least one resistance gene from the Albicidin Biosynthesis Gene Clusters into the plant to be protected.

EXAMPLE 1 – Materials and Methods

[0095] Bacterial strains and plasmids. The source of bacterial strains and their relevant characteristics are described in Table 1.

[0096] Media, antibiotics, and culture conditions. *X. albilineans* strains were routinely cultured on modified Wilbrink=s (MW) medium at 30□C without benomyl (Rott *et al.*, 1994). For long-term storage, highly turbid distilled water suspensions of *X. albilineans* were supplemented with glycerol to 15% (vol/vol) and frozen at B80□C. For *X. albilineans*, MW medium was supplemented with the following antibiotics as required at the concentrations indicated: kanamycin, 10 or 25 μg/ml; and rifampicin, 50 μg/ml. *E. coli* strains were grown on Luria-Bertani (LB) agar or in LB broth at 37□C and were maintained and stored according to standard protocols (Sambrook *et al.*, 1989). For *E. coli*, LB medium was supplemented with the following antibiotics as required at the concentrations indicated: kanamycin, 50 μg/ml; ampicillin, 50 μg/ml.

[0097] Bacterial conjugation. DNA transfer between *E. coli* donor (DH5_MCR/pAlb389 or pAC389.1, Table 1) and rifampicin-resistant *X. albilineans* recipients (*X.* strains AM10, AM12, AM13, AM36 and AM37, Table 1) was accomplished by triparental conjugation with plasmid pRK2073 as the helper as described previously (Rott *et al.*, 1996).

[0098] Assay of albicidin production. Albicidin production was tested by a microbiological assay as described previously (Rott *et al.*, 1996). Rifampicin and kanamycin exconjugants were spotted with sterile toothpicks (2-mm-diameter spots) onto plates of SPA

EXAMPLE 6 – Sequencing of the Tn*5* insertional site of eight tox⁻ mutants previously located in XALB1

[00109] Eight of the 45 *X. albilineans* Tox⁻ mutants complemented by cosmid pALB540 and/or cosmid pALB571 and previously described (Rott *et al.*, 1996) were further analyzed. All eight mutants contain a single Tn*5* insertion and correspond to the following *X. albilineans* strains: XaAM7, XaAM15, XaAM45, and XaAM52 which are complemented by pALB571 but not by pALB540; XaAM4, XaAM29 and XaAM40 which are complemented by both cosmids; and XaAM1 which is complemented by pALB540 but not by pALB571. The Tn*5* insertional site of each Tox⁻ mutant was sequenced from plasmids obtained following cloning in pBR325 or in pBluescript II KS (+) of the EcoRI fragments carrying Tn*5* and flanking sequence using the sequencing primer GUSN (5'tgcccacaggccgtcgagt3') SEQ ID No. 52 that annealed 135 bp downstream from the insertional sequence IS50L of Tn*5*-*gusA*. The sequence of the Tn*5* insertional site was compared with the 55,839 bp sequence containing XALB1 in order to determine the *alb* gene disrupted in each Tox⁻ mutant. *albI* is disrupted by the Tn*5* insertion in XaAM15 and XaAM45 at position 33443 and 34229, respectively (Figure 1). *albIV* is disrupted by the Tn*5* insertion in XaAM7 and XaAM52 at position 53704 and 53915, respectively. *albIX* is disrupted by the Tn*5* insertion in XaAM4, XaAM29 and XaAM40 at position 21653, 23444 and 24376, respectively. *alb XI* is disrupted by the Tn*5* insertion in XaAM1 at position 13301. These results are in accordance with the previous characterization of Tox⁻ mutants using Southern blot hybridization (Rott *et al.*, 1996), except for XaAM1. The Tn*5*-*gusA* insertion site of XaAM1 was previously located in DNA fragment A (Rott *et al.*, 1996) but results of this study showed that this site is located in DNA fragment J (Figure 1).

EXAMPLE 7 – Homology analysis of proteins potentially encoded by XALB1

[00110] Preliminary functional assignments of individual ORFs were made by comparison of the deduced gene products with proteins of known functions in the Genbank database. The results are set out in Table 3 below. Among the ORFs identified from the sequenced XALB1 gene cluster, we found (i) four genes, *albI* SEQ ID No. 20, *albIV* SEQ ID No. 23, *albVII* SEQ ID No. 17 and *albIX* SEQ ID No. 15, encoding PKS and/or NRPS modules; (ii) one carbamoyl transferase gene, *albXV* SEQ ID No. 5; (iii) two esterase genes, *albXI* SEQ ID No. 9 and *albXIII* SEQ ID No. 7; (iv) two methyltransferase genes, *albII* SEQ ID No. 21 and *albVI* SEQ ID No. 18; (v) two benzoate-derived products biosynthesis genes, *albXVII* SEQ ID No. 11 and *albXX* SEQ ID No.14; (vi) two putative albicidin biosynthesis regulatory genes, *albIII* SEQ ID No. 22 and *albVIII* SEQ ID No. 16; (vii) two putative albicidin resistance genes, *albXIV* SEQ ID No. 6 and *albXIX* SEQ ID No. 13; and (viii) two additional ORFs encoding proteins similar to transposition proteins, *albV* SEQ ID No. 19 and *albXVI* SEQ ID No. 4. No known

function was found in the database for *albX* SEQ ID No. 10 and *albXII* SEQ ID No. 8. The potential product of *albXVIII* SEQ ID No. 12 appeared to be a truncation of an enzyme with strong similarity to 4-amino-4-deoxychorismate lyase and branched-chain amino acid aminotransferase. Since the gene encoding the predicted product is roughly half the length of other such lyase or aminotransferase genes, *albXVIII* may be the result of a recombination event and may be non functional.

EXAMPLE 8 – The *alb* PKS and/or NRPS genes

[00111] The potential product of *albI*, designated AlbI SEQ ID No. 20, is a protein of 6879 aa with a predicted size of 755.9 kDa. This protein is very similar to the potential product of the *xabB* gene from *X. albilineans* strain Xa13 from Australia (Huang *et al.*, 2001), but it differs in length and size (See Table 4 below). XabB is a protein of 4801 amino acids with a predicted size of 525.7 kDa. Comparison of AlbI with XabB revealed that the N-terminal regions from Met-1 to Ile-4325 of both proteins are identical except for five amino-acids which are Tyr-3941, Pro-3952, Ala-4054, Ala-4271 and Gln-4284 in AlbI and His-3941, Ala-3952, Val-4054, Val-4271 and Glu-4284 in XabB. The same comparison revealed that the AlbI C-terminal region from Arg-6404 to the stop codon is 100% identical to the XabB C-terminal region from Arg-4326 to the stop codon.

[00112] The N-terminal region (from Met-1 to Asp-3235) of AlbI is 100% identical to the corresponding region in XabB which was previously described as similar to many microbial modular PKS (Huang *et al.*, 2001). This PKS region may be divided into three modules (Figure 2). Abbreviations used in the Figure are: A, adenylation; ACP, acyl carrier protein; AL, acyl-CoA ligase; C, condensation; KR, ß-ketoacyl reductase; KS, ß-ketoacyl synthase; NRPS, nonribosomal peptide synthase; PCP, peptidyl carrier protein; PKS, polyketide synthase; TE, thioesterase; HBCL, 4-hydroxybenzoate-CoA ligase. The question mark in the NRPS-2 domain indicates that this A domain is incomplete. The first module designated PKS-1 contains acyl-CoA ligase (AL) and acyl carrier protein (ACP1) domains. The second module designated PKS-2 contains ß-ketoacyl synthase (KS1) and ß-ketoacyl reductase (KR) domains followed by two consecutive ACP domains (ACP2 and ACP3). The third module designated PKS-3 contains a KS domain (KS2) followed by a PCP domain (PCP1). Apart from their very high similarity with XabB, these three PKS modules exhibited the highest degree of overall similarity with polyketide synthases SafB and PksM from *Myxococcus xanthus* and *Bacillus subtilis*, respectively (Table 4). The motifs characteristic of these domains are 100% identical to those of XabB which were previously aligned with those from other organisms (Huang *et al.*, 2001). The AL domain contains the conserved adenylation core sequence (SGSSG) and the ATPase motif (TGD). The three ACP domains contain a 4'-phosphopantetheinyl-binding cofactor box GxDS(IL), except that

Comparison of AlbVII sequence located upstream from residue 277 produced no significant alignment. AlbVII, like 4-hydroxybenzoate-CoA ligases, contains some conserved sequences characteristic of the A domain commonly found in peptide synthases (Table 6).

[00116] *albIX* encodes a protein of 1959 aa (AlbIX) with a predicted size of 218.4 kDa similar to non-ribosomal peptide synthases. Known conserved sequences, characteristic of the domains commonly found in peptide synthases (Marahiel *et al.*, 1997), were compared with those from AlbIX which forms two NRPS modules designated NRPS-6 and NRPS-7 (Tables 5, 6 and 7). NRPS-6 contains only one A and one PCP domain. NRPS-7 contains the three domains characteristic of NRPS modules (A-C-PCP) followed by a TE domain (Figure 2). Apart their very high similarity with XabB, NRPS-6 and NRPS-7 modules exhibited the highest degree of overall similarity and identity with non-ribosomal peptide synthases DhbF from *B. subtilis* and NosA from *Nostoc* sp. (Table 4).

EXAMPLE 9 – The alb carbamoyl transferase gene

[00117] *albXV* potentially encodes a protein of 584 aa with a predicted size of 65.2 kDa. This protein, AlbXV, is similar to BlmD, a carbamoyl transferase involved in bleomycin biosynthesis in *Streptomyces vertillus* (Du *et al.*, 2000), and to a probable carbamoyl transferase potentially expressed in *P. aeruginosa* (Table 4). High similarity of AlbXV with these proteins suggests that AlbXV is a carbamoyl transferase.

EXAMPLE 10 – The alb esterase genes

[00118] *albXI* potentially encodes a protein of 315 aa with a predicted size of 35.9 kDa. This protein, AlbXI, exhibits low similarity to SyrC, a putative thioesterase involved in syringomycin biosynthesis by *Pseudomonas syringae* (Zhang *et al.*, 1995), and to a potential hydrolase encoded by *Streptomyces coelicolor* (Table 4). Precise function of SyrC remains unknown but SyrC is similar to a number of thioesterases, including fatty acid thioesterases, haloperoxidases, and acyltransferases that contain a characteristic GxCxG motif. The corresponding SyrC domain GICAG is conserved in AlbXI which contains the sequence GWCQA, except that A replaces the last G, suggesting that AlbXI may be an esterase despite its low overall similarity with SyrC.

[00119] *albXIII* potentially encodes a protein of 317 aa with a predicted size of 34.5 kDa. This protein, AlbXIII, is similar to hypothetical proteins with unknown function from several bacteria including *Caulobacter crescentus* (Table 4). AlbXIII and these hypothetical proteins contain a GxSxG motif characteristic of serine esterases and thioesterases, the corresponding sequence in AlbXIII being GHSVG. In addition, AlbXIII presents a similarity with

the 2-acetyl-1alkylglycerophosphocholine esterase which hydrolyzes the platelet-activating factor in *Canis familiaris* (Table 4), suggesting that AlbXIII is an esterase.

EXAMPLE 11 – The alb methyltransferase genes

[00120] *albII* potentially encodes a protein of 343 aa (AlbII) with a predicted size of 37.7 kDa. *albII* is 100% identical to the *xabC* cistron, previously described as encoding an *O*-methyltransferase downstream *xabB* (Huang *et al.*, 2000a). This conclusion is based on the similarity of XabC with a family of methyltransferases that utilize S-adenosyl-L-methionine (SAM) as a co-substrate for *O*-methylation including TcmO protein from *Streptomyces glaucescens* (Huang *et al.*, 2000a). AlbII contains three highly conserved motifs of SAM-dependent methyltransferases, including the motif I involved in SAM binding (Figure 3). In the Figure, identical or similar amino acids (A=G; D=E; I=L=V) are shown in bold. Numbers indicate the position of the amino acid from the N-terminus of the protein. Abbreviations used in the Figure are: Sgl-TcmO and Sgl-TcmN, multifunctional cyclase-hydratase-3-*O*-Mtase and tetracenomycin polyketide synthesis 8-*O*-Mtase of *Streptomyces glaucescens*, respectively (accession number: M80674); Smy-MdmC, midecamycin-*O*-Mtase of *Streptomyces mycarofaciens* (accession number: M93958); Mxa-SafC, Saframycin *O*-Mtase of *Myxococcus xanthus* (accession number: U24657); Ser-EryG, erythromycin biosynthesis *O*-Mtase of *Saccharopolyspora erythraea* (accession number: S18533); Spe-DauK, carminomycin 4-O-Mtase of *Streptomyces peucetius* (accession number: L13453); Sal-DmpM, *O*-demethylpuromycin-*O*-Mtase of *Streptomyces alboniger* (accession number: M74560); Shy-RapM, rapamycin *O*-Mtase of *Streptomyces hygroscopicus* (accession number: X86780); Sav-AveD, avermectin B 5-*O*-Mtase of *Streptomyces avermitilis* (accession number: G5921167), Sar-Cmet, mithramycin *C*-methyltransferase of *Streptomyces argillaceus* (accession number: AF077869); AlbII, putative albicidin biosynthesis *C*-Methyltransferase of *Xanthomonas albilineans* (SEQ ID No. 27); identical to XabC, accession number: AF239749).

[00121] Comparison of AlbII with the Genbank database revealed that AlbII, besides 100% identity to XabC, exhibited the highest degree of overall identity with MtmMII, a *C*-methyltransferase from *Streptomyces argillaceus* (Table 4) involved in *C*-methylation of the polyketide chain for mithramycin biosynthesis, suggesting that AlbII is a *C*-methyltransferase. XabC was not compared by Birch and co-workers with MtmMII (Huang *et al.*, 2000a) because the MtmMII sequence was not available until recently in the Genbank database. The three highly conserved motifs in SAM methyltransfererases are also present in MtmMII (Figure 3), suggesting that AlbII is a *C*-methyltransferase SAM-dependent.

[00122] *albVI* potentially encodes a protein of 286 aa (AlbVI) with a predicted size of 32.1 kDa similar to several hypothetical protein from *Mycobacterium tuberculosis*

(Genbank accessions No. AAK46042, AAK48238, AAK44517, AAK46218) and from *S. coelicolor* (Genbank accession No. CAC03631). AlbVI is also similar to the tetracenomycine C synthesis protein (TcmP) of *Pasteurella multocida* (Table 4). Four highly conserved motifs in TcmP and other *O*-methyltransferases are also present in AlbVI (Figure 4), suggesting that AlbVI is an *O*-methyltransferase. In the Figure, identical or similar aa (A=G; D=E; I=L=V; K=R) are shown in bold. Numbers indicate the position of aa from the N-terminus of the protein. Abbreviations used in the Figure are: Sgl-tcmP, tetracenomycin C synthesis protein of *Streptomyces glaucescens* (accession number: C47127); Sme-PKS, putative polyketide synthase of *Sinorhizobium meliloti* (accession number: AAK65734); Pmu-tcmP: tetracenomycin C synthesis protein of *Pasteurella multocida* (accession number: AAK03406); Mtu-Omt: putative *O*-methyltransferase of *Mycobacterium tuberculosis* (accession number: AAK45444); Mlo-Hp: hypothetical protein containing similarity to *O*-methyltransferase of *Mesorhizobium loti* (accession number: BAB50127); Mtu-Hp1: hypothetical protein of *Mycobacterium tuberculosis* (accession number: AAK46042); Mtu-Hp2: hypothetical protein of *Mycobacterium tuberculosis* (accession number: AAK48238); Mtu-Hp3: hypothetical protein of *Mycobacterium tuberculosis* (accession number: AAK44517); AAK46218); Sco-Hp: hypothetical protein of *Streptomyces coelicolor* (accession number: CAC03631); AlbVI, putative albicidin biosynthesis *O*-Methyltransferase of *Xanthomonas albilineans* (this study). The three highly conserved motifs in SAM methyltransfererases are not present in AlbVI, indicating that SAM is not a co-substrate of AlbVI.

EXAMPLE 12 – The alb derived-benzoate products biosynthesis genes

[00123] alb*XVII* potentially encodes a protein of 716 aa with a predicted size of 79.8 kDa. This protein, AlbXVII, is very similar to the para-aminobenzoate (PABA) synthase from *Streptomyces griseus* (Table 4). This enzyme is required for the production of the antibiotic candicidin (Criado *et al.*, 1993).

[00124] *albXVIII* potentially encodes a protein of 137 aa with a predicted size of 15.0 kDa. This protein, AlbXVIII, is similar to the 4-amino-4-deoxychorismate lyase (ADCL) from *P. aeruginosa* (Table 4). The function of ADCL is to convert 4-amino-4-deoxychorismate into PABA and pyruvate. The length of AlbXVIII is smaller (Table 4) than the length of ADCL and the similarity of AlbXVIII with this protein starts only at residue 161. *albXVIII* is preceded by a small ORF encoding a sequence of 59 amino acids similar to the first 42 amino acids of ADCL from *P. aeruginosa*. These data suggest that *albXVIII* is probably a truncated form of *albXVIII* and probably not functional. *albXVIII* may, therefore, not be involved in albicidin biosynthesis. The region between *albXVII* and *albXVIII* was amplified by PCR from total DNA of *X. albilineans* Xa23R1 strain using primers ORFW (5'gcgagaggacaagctgctgc3') SEQ ID No. 53 and ORFY (5'cgttgaggatgcagcgctcg3') SEQ ID No. 54 and was sequenced. Resulting sequence data

involved in the hydroxylation of benzoyl CoA, a step of aerobic benzoate metabolism in *Azoarcus evansii*, but its function remains unknown (Mohamed *et al.*, 2001).

EXAMPLE 17 – Prediction of amino acid specificity of Alb NRPS modules

[00135] In NRPSs, specificity is mainly controlled by A domains which select and load a particular amino-, hydroxy- or carboxy-acid unit (Marahiel *et al.*, 1997). The substrate-binding pocket of the phenylalanine adenylation (A) domain of the gramicidin S synthetase (GrsA) from *Brevibacillus brevis* was recently identified by crystal structure analysis as a stretch of about 100 amino acid residues between highly conserved motifs A4 and A5 (Conti *et al.*, 1997). Based on sequence analysis of known A domains, in relation to the crystal structure of the GrsA (Phe)substrate binding pocket, similar models have been published to predict the amino acid substrate which is recognized by an unknown NRPS A domain (Challis *et al.*, 2000; Stachelhaus *et al.*, 1999). These models postulate specificity-conferring codes for A domains of NRPS consisting of critical amino acid residues putatively involved in substrate specificity. The model proposed by Marahiel and co-workers (Stachelhaus *et al.*, 1999) defined a signature sequence consisting of ten amino acids lining with the ten residues of the phenylalanine-specific binding pocket located at positions 235, 236, 239, 278, 299, 301, 322, 330, 331 and 517 in the GsrA (Phe) sequence (accession number: P14687). The model proposed by Townsend and co-workers (Challis *et al.*, 2000) uses only the first eight of these critical residues.

[00136] Preliminary specificity assignments of albicidin synthase AlbI, AlbIV, AlbVII and AlbIX NRPS modules were made by comparison of complete sequences between conserved motifs A4 and A5 with sequences in the Genbank database. The corresponding sequence of the AlbIV NRPS-5 module is most related to domain 5 of bacitracin synthase 3 (BA3) from *B. licheniformis* that was suggested to activate Asn (Konz *et al.*, 1997). Corresponding sequences of AlbI and AlbIX NRPS-1, NRPS-3, NRPS-6 and NRPS-7 modules, apart from their very high similarity with XabB, exhibited the highest degree of overall identity (39%) with the Blm NRPS2 module of the biosynthetic gene cluster for bleomycin from *S. verticillus* that specifies for β-Alanine (Du *et al.*, 2000). The corresponding sequence of AlbVII PKS-4 produced the highest significant alignment with acetate-CoA ligase from *Sulfolobus solfataricus* (Genbank accession number: AAK41550), aryl-CoA ligase from *Comamonas testosteroni* (Genbank accession number: AAC38458) and 4-hydroxybenzoate-CoA ligase from *R. palustris*. The sequence between motifs A4 and A5 of the AlbI NRPS-2 could not be significantly aligned with any sequence present in the Genbank database. Comparison of this sequence with the corresponding sequence of GrsA (Phe) revealed that parts of the putative core and structural "anchor" sequences of AlbI NRPS-2 are deleted (Figure 5), suggesting that the AlbI NRPS-2 substrate binding pocket is not functional. In the Figure, amino acids of the six Alb NRPSs and of Alb PKS-4 that are identical or similar to GrsA or Blm sequences (A=G; D=E;

BacC-M5 (Asn) (Bacitracin synthetase 3, accession number: AAC06348), TyrC-M1 (Asn) (Tyrocidine synthetase 3, accession number: AAC45930) and Asn code (Asn selectivity-conferring code defined by Marahiel and co-workers (Stachelhaus *et al.*, 1999). Amino acids of AlbI NRPS-1 and NRPS-3 signatures identical or similar to TyrB-M1 (Pro), VirS (Pro) and HVCL signatures (A=G; D=E; I=L=V; R=K) are shown in bold. Amino acids of AlbIX NRPS-6 and NRPS-7 signatures identical or similar to Vir (Pro) and Blm (ß-Ala) signatures (A=G; D=E; I=L=V; R=K) are shown in bold. Variability: 0 indicates invariant residues, +/- moderately variant residues and +-+ highly variant residues.

EXAMPLE 18 – Identification of putative promoters and putative terminators in XALB1

[00141] Putative rho independent terminators were identified downstream from *albIV* and *albXVI* using the Terminator program (Brendel and Trifonov, 1984), run with the Wisconsin Package™ GCG software (Figure 6). In the Figure, dashes indicate palindromic sequences. Symbols used in the Figure are: P, Primary structure value of putative terminator (minimum threshold value of 3.5 represents 95 percent of known, factor-independent, prokaryotic terminators); S, Secondary structure value of putative terminator. The presence of these terminators confirmed the proposed genetic organization of operons 1 and 3. A rho-independent terminator was identified in the intergenic region between *albXVII* and *albXVIII*, suggesting that the group of genes initially supposed to be organized in operon 4 may be in fact organized in two operons, operon 4 formed by *albXVII* and operon 5 by *albXVIII* B *albXX*. No putative rho independent terminator was found downstream from *albIX* and from *albXX*.

[00142] The 236 bp region between *albI* (operon 1) and *albV* (operon 2) is 100% identical to the sequence between *xabB* and *thp* genes that is assumed to contain a bidirectional promoter (Huang *et al.*, 2000a and 2001), suggesting that transcription of operon 1 and 2 is regulated by the same bidirectional promoter region (Huang *et al.*, 2001).

[00143] The 412 bp region comprised between *albX* (operon 3) and *albXVII* (operon 4) also contains a putative bidirectional promoter (Figure 7). In the Figure, the sequence of putative promoters are underlined, and putative ATG or TTG start codons are in bold. The closest matches (TTGACA-18x-TATAGT) to the consensus -35 (TTGACA) and -10 (TATAAT) sequences for *E. coli* σ^{70} promoters occurs 61 bp upstream from *albX* (operon 3). The closest matches (TTCAGA-19x-TATACA) to the consensus sequences for *E. coli* σ^{70} promoters occur 320 bp upstream from *albXVII* (operon 4). The region between *albXVII* and *albXVIII* lacks any apparent *E. coli* σ^{70} promoter. However, the sequence immediately upstream from *albXIX*, corresponding to the coding sequence of *albXVIII*, potentially contains an unidirectional promoter (Figure 7). The closest match (TTGCTC-19x-TATATT) to the consensus sequences for *E. coli* σ

70 promoters occurs 33bp upstream from *albXIX*. The presence of a terminator downstream from *albXVII* and of a promoter upstream from *albXIX* suggests that *albXVIII* is not transcribed and that *albXIX* and *albXX* form operon 5.

EXAMPLE 19 – Cloning of the XALB2 gene cluster

[00144] The 6 kb *Eco*R I fragment carrying Tn*5* and flanking sequence from strain AM37 was cloned in pBR325 and the obtained plasmid was designated pAM37 (Table 1). A 1.1 kb *Hin*d III-*Hin*d III DNA fragment from pAM37, named PR37 (Table 1), was labeled with ^{32}P and used to probe the 845 clones from the genomic library of *X. albilineans* strain Xa23R1, previously described (Rott et al., 1996). Eight new cosmids hybridized to this probe and restored albicidin production in mutant AM37. One of these cosmid, pALB389, carrying an insert of about 37 kb (Table 1), was used for complementation studies of the five mutants not complemented by pALB540 and pALB571. Cosmid pALB389 complemented mutants AM10 and AM37. Mutant AM10 was initially thought to be complemented by pALB639 (Rott et al., 1996). However, further complementation studies showed that mutant AM10 was not complemented by pALB639 and that only three mutants (AM12, AM13 and AM36) were complemented by pALB639 containing the third genomic region XALB3 involved in albicidin production. A 3 kb *Eco*RI- *Eco*RI DNA fragment from pALB389 that hybridized with probe PR37 was sub-cloned in pUFR043 (Table 1). The resulting plasmid pAC389.1 complemented mutants AM10 and AM37, confirming that the second region involved in albicidin production, XALB2, was present in the 3 kb insert of pAC389.1.

EXAMPLE 20 – Cloning of the XALB3 gene cluster

[00145] Cosmid pALB639, carrying an insert of 36 kb (Rott *et al.*, 1996; Table 1) was used as a probe to compare the *Eco*RI restriction profiles of *X. albilineans* strain Xa23R1 with those of mutants AM12, AM13 and AM36 which were supposed to be mutated in the XALB3 gene cluster. An 11 kb band which was found in strain Xa23R1 but not in the three mutants was supposed to contain the XALB3 gene cluster. A 9.7 kb *Eco*RI DNA fragment purified from cosmid pALB639 also used as a probe in Southern blot analyse revealed the same 11 kb band. This 9.7 kb *Eco*RI DNA fragment was sub-cloned in pUFR043 (Table 1) and the resulting plasmid pAlb639A complemented mutants AM12, AM13 and AM36. The third region involved in albicidin production, XALB3, was therefore present in the 9.7 kb insert of pAlb639A.

EXAMPLE 24 - Heterologous production of albicidin in fast growing *Xanthomonas axonopodis* pv. Vesicatoria.

[00154] This example illustrates the construction of a heterologous expression system harboring the three XALB regions, its transfer into a fast growing host, *Xanthomonas axonopodis* pv. vesicatoria and the subsequent production of a potent toxin with an antibiotic activity similar to that of albicidin. This work is a milestone in the validation of the albicidin biosynthesis model because it gives experimental evidence that the entire biosynthetic machinery required for albicidin biosynthesis has been identified, cloned, sequenced and transferred into an heterologous host, driving the production of albicidin. Cosmid pALB571 which covers the complete sequences of operons 1 and 2 was used to transfer operons 1 and 2 (Figure 1). Operons 3 and 4 (from pALB540), XALB2 (from pAC389.1) and XALB3 (from pEV639) were subcloned into a single plasmid, pOp3-4/XALB2-3 (see below). Plasmid pOp3-4/XALB2-3 derived from shuttle vector pLAFR3 that carries one selective gene for resistance to tetracyclin and that belongs to incompatibility group IncP (Table 1). Cosmid pALB571 derived from shuttle vector pUFR043 that carries two selective genes for resistance to kanamycine and gentamycine and that belongs to incompatibility group IncW (Table 1).

Sub-cloning of operons 3 and 4 and XALB2 and XALB3 regions into a single plasmid (Figure 12).

[00155] A 2,787 bp *Bam*HI – *Pst*I fragment from pALB540, corresponding to a portion of operon 4, was subcloned into pBCKS(+), yielding pBC/Op4Δ (step 1). A *Xho*I site was introduced into this vector immediately upstream from the *Bfr*I site by directed mutagenesis. Mutagenesis was performed with primers XhoIAlb anticodant 5'cgccttaagcagctcgagtagactgcaatc3' and XhoIAlbcodant 5'gattgcagtctactcgagctgcttaaggcg3' and yielded plasmid pBC/Op4ΔXhoI (step 2). The 2,986 bp *Eco*RI fragment from pAC389.1 (containing XALB2) was then subcloned into pBC/Op4ΔXhoI, yielding pBC/Op4Δ/XALB2 (step 3). A 10,762 bp *Bfr*I fragment from pALB540 and containing complete operon 3 and the beginning of operon 4 was subcloned into pBC/Op4Δ/XALB2 yielding pBC/Op3-4/XALB2 (step 4). The 2,615 bp *Sal*I fragment from pEV639 (containing XALB3) was subcloned into pBKS, yielding pBKS/XALB3 (step 5). The *Sal*I site located on the *Kpn*I side of the polylinker was then destroyed and substituted by a *Xho*I restriction site by directed mutagenesis. This mutagenesis was performed with primers XhoSalXaHTPGR 5'gcttatcgataccctcgaggaaggcgatatcg3' and XhoSalXaHTPGF 5'cgatatcgccttcctcgagggtatcgataagc3', yielding pBKS/XALB3XhoI (step 6). Finally, the *Xho*I cassette of pBC/Op3-4/XALB2 was subcloned into the *Sal*I restriction site of pBKS/XALB3XhoI, yielding pBKS/Op3-4/XALB2-3 (step 7). This construct harbours an *Xho*I cassette containing complete operons 3 and 4 from XALB1, *albXXI* from XALB2 and *albXXII*

from XALB3. An *Xho*I site was added to the *Bam*HI site of the pLAFR3 shuttle vector polylinker using the adaptor AdApTBamHIXhoI 5'gatcgctcgagc3', yielding pLAFR3XhoI (step 8). The *Xho*I cassette from pBKS/Op3-4/XALB2-3 was then cloned into pLAFR3XhoI, yielding pOp3-4/XALB2-3 (step 9). This last construct was used, along with pALB571 (operons 1 and 2), for heterologous expression of albicidin in *X. axonopodis* pv. vesicatoria.

Albicidin production assays

[00156] The four combinations of plasmids (i.e. pUFR043–pLAFR3, pUFR043-pOp3-4/XALB2-3, pAlb571-pLAFR3 and pAlb571-pOp3-4/XALB2-3) were transferred into *X. axonopodis* pv. vesicatoria strain Xcv 91-11BR1 by triparental mating. Exconjugant clones resistant to tetracycline and kanamycin were isolated. Assays for albicidin production were performed with these exconjugants clones using the same method described in Example 1 except that tetracycline (12 mg/ml) and/or kanamycin (50 mg/ml) were added to SPA medium. Tetracycline and kanamycin resistant *E. coli* clones, DH5αKT and DH5αAlb^{r}KT (Table 1), were used as tester strains to evaluate albicidin production to ensure that growth inhibition was not due to the presence of these two antibiotics in SPA medium. Both clones, DH5αKT and DH5αAlb^{r}KT, are tetracycline and kanamycin resistant because they carry plasmids pLAFR3 and pUFR043. The albicidin resistant DH5αAlb^{r}KT clone derived from strain DH5αAlb^r (Table 1) which is a spontaneous albicidin resistant clone isolated in a growth inhibition zone produced by *X. albilineans* strain Xa23R1.

[00157] Without antibiotics in the SPA medium, growth of clones DH5αKT and DH5αAlb^{r}KT was not inhibited in all assays performed with the different *X. axonopodis* pv. vesicatoria exconjugants. Surprisingly, when kanamycin was present in the SPA medium, growth of both DH5αKT and DH5αAlb^{r}KT was inhibited in all assays performed with the *X. axonopodis* pv. vesicatoria exconjugants. These results suggested that, in the presence of kanamycin, all *X. axonopodis* pv. vesicatoria exconjugants produced an antibiotic inhibiting growth of *E. coli*. Because exconjugants containing only empty vectors (pUF043 and pLAFR3) induced inhibition of *E. coli*, this antibiotic did not result from the expression of XALB1, XALB2 and/or XALB3. Additionally, there was no cross resistance between this antibiotic and albicidin. When tetracyclin was present in the bioassay medium, but not kanamycin, growth of the albicidin resistant clone (DH5αAlb^{r}KT) was not inhibited by any of the exconjugants. In contrast, growth of the albicidin susceptible *E. coli* strain (DH5αKT) was inhibited by the exconjugants harbouring pALB571 and pOp3-4/XALB2-3 plasmids, but not by exconjugants harbouring the other three combinations of plasmids (Table 10). This result suggested that expression of the XALB1, XALB2 and XALB3 regions in *X. axonopodis* pv. vesicatoria (harbouring pALB571 and pOp3-4/XALB2-3 plasmids) led to the production of an albicidin-like antibiotic. This product inhibited growth of an albicidin sensitive *E. coli* (DH5αKT) and had no effect on the growth of an albicidin resistant clone

(DH5αAlb^{r}KT).

[00158] Preliminary results indicated that pLAFR3 derived plasmids were relatively unstable in the absence of tetracycline in the culture medium, suggesting that genes carried by pOp3-4/XALB2-3 were not expressed when *X. axonopodis* pv. vesicatoria exconjugants pALB571/pOp3-4/XALB2-3 were grown without tetracycline. Consequently, these exconjugants did not produce the albicidin-like compound in absence of any antibiotic in the culture medium (Table 10). Preliminary results also indicated that pUFR043 derived plasmids are relatively stable in *X. axonopodis* pv. vesicatoria in absence of antibiotic selection, suggesting that genes carried by pALB571 are expressed when *X. axonopodis* pv. vesicatoria exconjugants pALB571/pOp3-4/XALB2-3 were grown on media without kanamycin. Consequently, these exconjugants produced the albicidin-like compound on SPA containing only tetracyclin.

[00159] Two *E. coli* DH5αKT clones, that spontaneously grew within the growth inhibition zone of a *X. axonopodis* pv. vesicatoria pALB571-pOp3-4/XALB2-3 exconjugant on SPA + tetracycline medium, were isolated and tested for resistance to albicidin. No growth inhibition was observed when these clones were used as tester strains in an albicidin production assay performed with *X. albilineans* Xa23R1. These results showed that cross-resistance occurs between the albicidin-like product of *X. axonopodis* pv. vesicatoria and albicidin produced by *X. albilineans*, suggesting that both molecules are similar. Comparison of chemical characteristics of the two molecules will, however, be necessary to confirm that the two molecules are identical.

[00160] The invention includes the isolation and sequencing of a region of 55,839 bp from *X. albilineans* strain Xa23R1 containing the major gene cluster XALB1 involved in albicidin production. Analysis of this region allowed us to predict the genetic organization of the gene cluster XALB1 which contains 20 ORFs grouped in four or five operons (Figure 1). Because *albXVIII* is a truncated gene, XALB1 genes may be organized in five operons. Therefore, we will from now on consider *albXVII* as part of operon 4 and *albXIX* and *albXX* as part of operon 5. Similar operon-type organizations for antibiotic biosynthesis clusters are well known and have been postulated to facilitate cotranslation of genes within the operon to yield equimolar amounts of proteins for optimal interactions to form the biosynthesis complexes (Cane, 1997). Overlapping genes involved in the same process are also quite common in bacteria (Normark *et al.*, 1983).

[00161] Previous results of transposon mutagenesis and complementation studies (Rott *et al.*, 1996; Rott, unpublished results) are in accordance with the predicted genetic organization of XALB1 described in this study, and allowed us to establish that operons 1, 2 and 3 are involved in albicidin biosynthesis: (I) Tox$^-$ mutants with a Tn*5-gusA* insertion site located in DNA fragments B, C, G and D were complemented by cosmid pALB571 and not by cosmid pALB540, confirming that cosmid pALB571 potentially contains the entire operon 1; (ii) Tox$^-$ mutants with a Tn*5-gusA* insertion site located in DNA fragments A and H were complemented

like *xabB* (Huang *et al.*, 2001), use TTG as a start codon, which may impose post-transcriptional control of the rate of gene product formation (McCarthy and Gualerzi, 1990).

[00165] The predicted genetic organization of operons 1 and 2 presents similarities with the organization of the region involved in albicidin production in strain Xa13 of *X. albilineans* from Australia (Huang *et al.* 2000a, Huang *et al.*, 2001). This latter region also contains two divergent operons involved in albicidin production, one comprising the *xabB* gene (similar to *albI*, but with a large deletion) and the *xabC* gene (100% identical to *albII*) and the other containing *thp* gene (100% identical to *albV*). In addition, the sequence between the two operons in strain Xa13 is 100% identical to the sequence between operons 1 and 2, indicating that both clusters are controlled by the same bidirectional promoter. However, transposon mutagenesis studies of Xa13 showed no evidence of another cistron downstream of *xabC* that may be involved in albicidin production (Huang *et al.*, 2000a), suggesting that the Xa13 *xab* operon differs from the Xa23R1 operon 1, which contains two additional genes downstream from *albII* that are potentially involved in albicidin production (*albIII* and *albIV*; refer Figure 1).

[00166] Homology analysis revealed that four NRPS and/or PKS genes are present in XALB1 (Figure 2), and these genes may be involved in the biosynthesis of the albicidin polyketide-polypeptide backbone (*albI*, *albIV*, *albVII* and *albIX*). NRPS and PKS enzymes are generally organized into repeated functional units known as modules, each of which is responsible for a discrete stage of polyketide or polypeptide chain elongation (Cane and Walsh, 1999). Each PKS or NRPS module is made up of a set of three core domains, two of which are catalytic and one of which acts as a carrier, and together are responsible for the central chain-building reactions of polyketide or polypeptide biosynthesis. Both PKS and NRPS core domains utilize analogous acyl-chain elongation strategies in which the growing chain, tethered as an acyl-S-enzyme to the flexible 20 Å long phosphopantetheinyl arm of an acyl carrier protein (ACP) or peptidyl carrier protein (PCP) domain, acts as the electrophilic partner that undergoes attack by a nucleophilic chain-elongation unit, a malonyl- or aminoacyl-S-enzyme derivative, respectively, itself covalently bound to a downstream ACP/PCP domain. In the case of a PKS, the fundamental chain-elongation reaction, a C-C bond-forming step, is mediated by a ketosynthase (KS) domain that catalyzes the transfer of the polyketide acyl chain to an active-site cysteine of the KS domain, followed by condensation with the methylmalonyl- or malonyl-S-ACP by a decarboxylative acylation of the malonyl donor unit. An additional essential component of the core PKS chain-elongation apparatus is an associated acetyltransferase (AT) domain, which catalyzes the priming of the donor ACP sidearm with the appropriate monomer substrate, usually methylmalonyl- or malonyl-CoA. The comparable core domains of an NRPS biosynthetic module function in a chemically distinct but architecturally and mechanistically analogous fashion. In the latter case, the key chain-building reaction, a C-N bond-forming reaction, involves the generation of the characteristic peptide bond by nucleophilic attack of the amino group of an amino acyl-S-PCP

deleted domain may be not functional. In model 1, (Figure 9A), (i) the PKS-1 module alone is responsible for the initiation of the acyl-chain assembly, (ii) PKS-4 (HBCL) interacts with PKS-2 and PKS-3 as an AT domain to allow acyl transfer and (iii) NRPS-5 interacts with only NRPS-2. In model 2 (Figure 9B) two different modules, PKS-1 and PKS-4, are responsible for this initiation step. Model 2 leads to the biosynthesis of four different polyketide-polypetide backbones; in this model (i) PKS-1 (AL) and PKS-4 (HBCL) are in competition for initiation of albicidin precursors; (ii) a separate AT enzyme (potentially AlbXIII) interacts with PKS-2 and PKS-3 to allow acyl transfer; (iii) NRPS-5 interacts with NRPS-2; and (iv) NRPS-5 and NRPS-6 are in competition for interaction with NRPS-4.

[00169] Both models are based on the fact that PKS-1 contains the AL and ACP1 domains, and PKS-4 shows homology with the hydroxybenzoate-CoA ligases. In other PKS systems, an N-terminal AL domain is involved in the activation and incorporation of an 3,4-dihydroxycyclo hexane carboxylic acid, a 3-amino-5-hydroxybenzoic acid or a long-chain fatty acid as a starter (Aparicio *et al.*, 1996; Motamedi and Shafiee, 1998; Tang *et al.*, 1998; Duitman *et al.*, 1999). PKS-4 may be also involved in the activation and incorporation of hydroxy-benzoate but this latter domain lacks any ACP or PCP domain, suggesting that PKS-4 is responsible for initiation of the acyl-chain assembly (Figure 9B) onto one of the three ACP domains of AlbI (ACP1, ACP2 or ACP3). The 277 amino-acids preceding the PKS-4 module in AlbVII may be necessary for the intercommunication between AlbVII and AlbI. The presence of two different PKS modules potentially involved in the initiation of the acyl-chain assembly suggests a competition of these two modules for the initiation of two different albicidin polyketide-polypeptide backbones, and this could contribute to the production of multiple, structurally related albicidins by the same cluster XALB1. Production of two different components, one initiated by PKS-4 containing an additional aromatic ring due to incorporation of hydroxybenzoate, may explain why partial characterization of albicidin indicated the presence of a variable number (three or four) of aromatic rings (Huang *et al.*, 2001).

[00170] In AlbI, PKS-1 is followed by the PKS-2 module which contains a KS domain and a KR domain upstream from two ACP domains (ACP2 and ACP3) and it lacks any discernable AT domain. Tandem ACP domains are unusual within PKS modules but have been shown to occur in the biosynthesis of several fungal and bacterial polyketide synthases (Mayorga and Timberlake, 1992; Yu and Leonard, 1995; Takano *et al.*, 1995; Albertini *et al.*, 1995). However, the significance of the tandem ACP domains in these systems has not been solved yet. In our model 2, one of the tandem ACP (ACP2 or ACP3) may interact with PKS-4 for the initiation of an acyl-chain assembly (Figure 9B). The absence of an AT domain in the PKS-2 module suggests that a separate AT domain is indispensable for the elongation of the acyl-chain initiated by this module. Separate AT enzymes encoded elsewhere in the genome were described in other systems for two PKS modules lacking AT domains: malonyl-CoA transacyclase gene

production of four components structurally very different. The polyketide moieties of the acyl chains initiated by the AlbI AL domain or by the AlbVII HBCL domain may be very different. The polyketide moiety of acyl chains initiated by the AlbVII HBCL domain may be shorter and may contain an additional aromatic ring. The presence of four structuraly different metabolites may explain the difficulty observed by Birch and Patil (1985a) to purify albicidin and to determine its chemical structure.

[00175] Homology analysis also revealed that AlbI NRPS-1 and 3 and AlbIX NRPS-6 and 7 specify unusual substrates which seem to contain an amino group and a carboxylate group but to be different from α-amino acids and β-alanine. Identification of several aromatic rings in albicidin (Huang *et al.*, 2001) suggested that NRPS-1, -3, -6 and -7 are involved in incorporation of aromatic substrates. By analogy with the Asp235Val alteration in the β-Ala specificity-conferring code (Du *et al.* 2000), the Asp235Ala alteration in the NRPS-1, -3, -6 and -7 signatures could be consistent with a large distance between the amino group and the carboxylate group in the substrate specified by these modules. Based on this hypothesis, we suggest that operons 3, 4 and 5 are involved in the biosynthesis of two aromatic substrates: the para-aminobenzoate potentially synthesized by AlbXVII (para-aminobenzoate synthase), and the carbamoyl benzoate potentially synthesized by AlbXX (hydroxybenzoate synthase) and AlbXV (carbamoyl transferase). Incorporation of these nonproteinogenic substrates may explain why albicidin is insensitive to proteases (Birch and Patil, 1985a).

[00176] According to biosynthesis model 1 leading to the biosynthesis of only one polyketide-polypeptide albicidin backbone that may correspond to the major component produced by XAlb1, we propose a model allowing prediction of the composition and the structure of albicidin (Figure 11). In the Figure, NRPS and PKS domains are abbreviated as follows: A, adenylation; ACP, acyl carrier protein; AL, acyl-CoA ligase; C, condensation; KR, ß-ketoreductase; KS, ß-ketoacyl synthase; PCP, peptidyl carrier protein. C atoms of albicidin-backbone are numbered 1 to 38. Bold methyl groups correspond to methylation of the albicidin backbone by AlbII or AlbVI. In this model, albicidin biosynthesis is initiated by loading of an acetyl-CoA by PKS-1 (step 1), and the chain product is elongated by incorporation of (I) malonyl-CoA by PKS-2 and PKS-3 (steps 2 and 3), (ii) para-aminobenzoate or carbamoyl benzoate by NRPS-1 and NRPS-3 (steps 4 and 6), (iii) asparagine by NRPS-2 coupled to NRPS-5 (step 5) and (iv) para-aminobenzoate or carbamoyl benzoate by NRPS-6 and NRPS-7 (steps 7 and 8). The presence of the KR domain in the PKS-2 module may lead to the formation of an hydroxyl group at the C_2 atom of the albicidin backbone. This hydroxyl group might be methylated by AlbVI (*O*-methyltransferase). The acyl chain may also be modified by AlbII (*C*-methyltransferase) at C_{13} or C_{14}.

[00177] The chemical composition ($C_{40}O_{15}N_6H_{35}$), the molecular weight (839), and the structure of the putative XALB1 product are in accordance with the partial characterization of albicidin published by Birch and Patil (1985a) which indicated that albicidin contains approximately 38 carbon atoms and a carboxylate group and that the molecular weight of albicidin was about 842. The presence of two ester linkages in our predicted albicidin structure is also in accordance with the fact that albicidin is detoxified by the AlbD esterase (Zhang and Birch, 1997). However, an unpublished albicidin analysis cited by Huang *et al.* (2001) indicated the presence of (I) two OCH3 groups and not one as in our predictive albicidin structure, (ii) one CN linkage and not eleven as in our predictive albicidin structure and (iii) a trisubstituted double bond that is not present in the putative XALB1 product.

[00178] In conclusion, homology analysis of XALB1 revealed unprecedented features for hybrid polyketide-peptide biosynthesis in bacteria involving a *trans*-action of four PKS and seven NRPS separate modules which could contribute to the production of multiple, structurally related polyketide-peptide compounds by the same gene cluster. Characterization of the full chemical structure of albicidin may be necessary to validate these models. Four NRPS modules seem to activate a very unusual substrate. Over- expression and purification of A domains from these four NRPS modules will be necessary to examine their substrate specificities. Substrate specificity of each A domain will therefore be determinated by analysis of the ATP-PPi exchange reaction with different substrate putatively incorporated into albicidin. Investigating albicidin backbone biosynthesis will be of great interest because such information adds to the limited knowledge as to how PKS and NRPS interact and how they might be manipulated to engineer novel molecules, and may explain how *X. albiltneans* produces several structurally related, toxic compounds.

[00179] Cloning and sequencing of XALB2 showed that the same phosphopantetheinyl transferase is required for albicidin production in an *X. albiltneans* strain from Florida and in an *X. albilineans* strain from Australia (Huang *et al.*, 2000b), explaining the precedented results showing that strain LS156 mutated in *xabA* (100% identical to *albXXI*) was not complemented by pALB540, pALB571 and pALB639 (Rott *et al.*, 1996). Mutant LS156 was shown to be complemented by a construction containing the coding sequence of *xabA* in fusion with *lacZ*, revealing that *xabA* is required for albicidin production and that no other cistron downstream from *xabA* was involved in albicidin production (Huang *et al.*, 2000b). However, this complementation study did not allow determination of whether *xabA* is transcribed as a part of a larger operon. Here we disclose the complementation of mutant AM37 with a 2986 bp insert from *X. albilineans* containing *albXXI* (100% identical to *xabA*), confirming that *albXXI* is involved in albicidin biosynthesis and indicating that the promoter of *albXXI* is present in the 2986 bp insert and that *albXXI* is not expressed as part of a operon.

Table 1 : Bacterial strains and plasmids used in this study

	Relevant characteristics[a]	Reference or source
Strains		
E. coli		
DH5α	F-/80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) supE44 thi-1 *gyrA96 relA1*	Gibco-BRL
DH5α MCR	DH5α*mcrA mcrBC mrr*	"
Xcv 91-11B	Wild type strain of *Xanthomonas axonopodis* pv. vesicatoria from tomato (race 3)	Astua-Monge *et al.*, 2000
Xcv 91-11BR1	Spontaneous Rif[r] derivative of Xcv 91-11B	This study
DH5αKT	*Escherichia coli* DH5α strain transformed by both pUFR043 and pLAFR3 plasmids	"
DH5αAlb[r]	Spontaneous Alb[r] derivative of DH5α	"
DH5αAlb[r]KT	DH5αAlb[r] transformed by both pUFR043 and pLAFR3 plasmids	"
Plasmids		
PBR325	Tc[r], Ap[r], Cm[r]	Gibco-BRL
pBCKS (+)	Cm[r]	Stratagene
pBluescript II KS (+)	Ap[r]	"
PRK2073	PRK2013 derivative, Km[s] (*npt*::Tn*7*), Sp[r], Tra[+], helper plasmid	Leong *et al.*, 1982
pUFR043	IncW Mob[+] LacZα Gm[r], Km[r], Cos	De Feyter and Gabriel, 1991
pAlb540	47 kb insert from Xa23R1 in pUFR043, Gm[r], Km[r]	Rott *et al.*, 1996
pAlb571	36.8 kb insert from Xa23R1 in pUFR043, Gm[r], Km[r]	"
PAlb639	36 kb insert from Xa23R1 in pUFR043, Gm[r], Km[r]	"
pAM15.1	24 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM15 in pBR325, Km[r], Tc[r], Ap[r], Cm[r]	"
pAM40.2	11 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM40 in pBR325, Km[r], Tc[r], Ap[r], Cm[r]	"
pAM45.1	12 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM45 in pBR325, Km[r], Tc[r], Ap[r], Cm[r]	"
pAM12.1	13 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM12 in pBR325, Km[r], Tc[r], Ap[r], Cm[r]	A
PAM36.2	9 kb*Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM36 in pBR325, Km[r], Tc[r], Ap[r], Cm[r]	A
pAlb389	37 kb insert from Xa23R1 in pUFR043, Gm[r], Km[r]	This study
pAC389.1	2.9 kb insert from Xa23R1 in pUFR043, Gm[r], Km[r]	"
PAlb639A	9.4 kb insert from Xa23R1 in pUFR043, Gm[r], Km[r]	"
PEV639	2.6 kb *Sal* I insert from Xa23R1 in pUFR043, Gm[r], Km[r]	"
pBC/A[r]	7.5 kb *Kpn* I fragment carrying a part of fragment A from pAlb571 in pBCKS (+), Cm[r]	"
pBC/AF	15.2 kb *Eco*R I fragment carrying fragments A and F from pALB540 in pBCKS (+), Cm[r]	"
pBC/B	11.0 kb *Kpn* I fragment B from pAlb571 in pBCKS (+), Cm[r]	"
pBC/C	6.0 kb *Kpn* I fragment C from pAlb571 in pBCKS (+), Cm[r]	"
pBC/E	2.8 kb *Kpn* I fragment E from pAlb571 in pBCKS (+), Cm[r]	"
pBC/F	2.5 kb *Kpn* I-*Eco*R I fragment F from pAlb571 in pBCKS (+), Cm[r]	"
pBC/G	1.9 kb *Eco*R I fragment G from pAlb571 in pBCKS (+), Cm[r]	"

Table 1 : Bacterial strains and plasmids used in this study

	Relevant characteristics[a]	Reference or source
pBC/I	1.4 kb *Kpn* I-*Eco*R I fragment I from pAlb571 in pBCKS (+), Cm^r	"
pBC/J	0.6 kb *Eco*R I fragment J from pALB540 in pBCKS (+), Cm^r	"
pBC/K	4.7 kb *Eco*R I fragment K from pALB540 in pBCKS (+), Cm^r	"
pBC/L	0.4 kb *Eco*R I fragment L from pALB540 in pBCKS (+), Cm^r	"
pBC/N	7.7 kb *Eco*R I fragment N from pALB540 in pBCKS (+), Cm^r	"
pUFR043/D=	2.2 kb *Eco*R IB*Sau*3A I fragment carrying a part of fragment D from pAlb571 in pUFR043	"
pAM1	5 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM1 in pBluescript II KS (+), Km^r, Ap^r	"
pAM4	12 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM*4* in pBluescript II KS (+), Km^r, Ap^r	"
pAM7	6 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM*7* in pBluescript II KS (+), Km^r, Ap^r	"
pAM10	7 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM10 in pBluescript II KS (+), Km^r, Ap^r	"
pAM29	10 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM29 in pBluescript II KS (+), Km^r, Ap^r	"
pAM37	6 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM37 in pBR325, Km^r, Tc^r, Ap^r, Cm^r	"
pAM52	5 kb *Eco*R I fragment carrying Tn*5* and flanking sequences of mutant AM52 in pBluescript II KS (+), Km^r, Ap^r	"
PLAFR3	IncP, Mob+, *LacZ*α, Tc^r, *cos*	Staskawicz *et al.*, 1987
PLAFR3XhoI	pLAFR3 with a *Xho*I site added to the *Bam*HI site using an adaptator	This study
pBC/Op4Δ	*Bam*HI-*Pst*I fragment from pALB540 cloned between *Bam*HI and *Pst*I sites of pBCKS(+)	"
pBC/Op4ΔXhoI	PBC/Op4Δ with a *Xho*I site created by directed mutagenesis upstream from the *Bfr*I site	"
pBC/Op4Δ/XALB2	*Eco*RI DNA fragment from pAC389.I cloned into the *Eco*RI site of pBC/Op4ΔXhoI	"
pBC/Op3-4/XALB2	*Bfr*I DNA fragment from pALB540 cloned into the *Bfr*I site of pBC/Op4Δ/XALB2	"
pBKS/XALB3	*Sal*I DNA fragment from pEV639 cloned into the *Sal*I site of pBluescript II KS (+)	"
pBKS/XALB3XhoI	pBKS/XALB3 with a *Xho*I site created by directed mutagenesis to substitute the *Sal*I site located on the *Kpn*I side of the polylinker	"
pBKS/Op3-4/XALB2-3	*Xho*I DNA fragment from pBC/Op3-4Δ/XALB2 cloned into the *Sal*I site of pBKS/XALB3XhoI	"
pOp3-4/XALB2-3	*Xho*I DNA fragment from pBKS/Op3-4/XALB2-3 cloned into the *Xho*I site of pLAFR3XhoI	"
pEValbXXII	*albXXII* in fusion with LacZ in pUFR043, Gm^r, Km^r	"
pEVHtpG	*E. coli htpG* in fusion with LacZ in pUFR043, Gm^r, Km^r	"
PGemT	ColE1 replicon, Ap^r, LacZα, single 3'-T overhangs at the insertion site	Promega
PGemT/albXXII	PCR fragment containing *albXXII* cloned into pGemT	This study
PGemT/albXXII bis	*Bgl*II-*Sal*IDNA fragment from pBKS/XALB3 cloned between the *Bgl*II and *Sal*I sites of pGemT/albXXII	"
PGemT/HtpG	PCR fragment containing the *E. coli htpG* gene cloned into pGemT	"

Table 4: Summary of results obtained from BLAST analyses.

Putative Alb protein	No. of aa residues	Protein homolog	Origin	Genbank accession #	Score	Expect	Identities	Positives	Gaps
AlbVII	765	4-hydroxybenzoate-CoA ligase (539 aa)	*Rhodopseudomonas palustris*	AAA62604	203 bits (513)	5e-51	156/492 (31%)	242/492 (48%)	31/492 (6%)
AlbVIII	330	SyrP Like (339 aa) SyrP (353 aa)	*S. verticillus* *Pseudomonas syringae*	AF210249 AAB63253	245 bits (619) 182 bits (458)	6e-64 5e-45	130/309 (42%) 106/306 (34%)	182/309 (58%) 155/306 (50%)	2/309 (0%) 4/306 (1%)
AlbIX	1959								
NRPS-6		XabB (4801 aa) DhbF (1278 aa)	*X. albilineans* *B. subtilis*	AAK15074 CAB15186	481 bits (1239) 354 bits (908)	e-135 1e-96	286/608 (47%) 222/608 (36%)	374/608 (61%) 341/608 (55%)	23/208 (3%) 21/608 (3%)
NRPS-7		XabB (4801 aa) NosA (4379 aa)	*X. albilineans* *Nostoc* sp.	AAK15074 AF204805	874 bits (2258) 551 bits (1420)	0.0 e-155	515/1110 (46%) 388/1148 (33%)	682/1110 (61%) 583/1148 (49%)	52/1110 (4%) 84/1148 (7%)
AlbX	83	Hypothetical protein (72 aa) MbtH (71 aa)	*P. aeruginosa* *M. tuberculosis*	AAG05800 CAB08480	75.6 bits (185) 59 bits (142)	1e-13 9e-09	34/61 (55%) 25/55 (45%)	44/61 (71%) 37/55 (66%)	- -
AlbXI	315	SyrC (433 aa) Hydrolase (261 aa)	*P. syringae* *S. coelicolor*	AAA85161 CAA16200	34.4 bits (78) 34 bits (77)	1.9 2.9	23/93 (24%) 19/60 (31%)	40/93 (42%) 30/60 (49%)	- -
AlbXII	451	BoxB (473 aa	*Azoarcus evansii*	AAK00599	293 bits (751)	3e-78	174/448 (38%)	243/448 (53%)	12/448 (2%)
AlbXIII	317	Hypothetical protein (335 aa) Plasma PAF acetylhydrolase (444 aa)	*Caulobacter crescentus* *Canis familiaris*	AAK25001 AAC48484	99.5 bits (247) 37.5 bits (86)	5e-200	88/296 (29%) 43/156	125/296 (41%) 56/156	5/296 (1%) 44/156 (28%)
AlbXIV	496	Putative trans-membrane efflux protein (505 aa) AlbF, putative albicidin efflux pump (496 aa)	*S. coelicolor* *X. albilineans*	CAB90983 AF403709	225 bits (574) 736 bits (1900)	0	154/465 (33%) 496/496 (100%)	240/465 (51%) 496/496 (100%)	8/465 (1%) -
AlbXV	584	Probable carbamoyl transferase (585 aa) BlmD (545 aa)	*P. aeruginosa* *S. verticillus*	AAG08390 AAG02370	201 bits (513) 192 bits (506)	1e-50 1e-47	158/458 (34%) 149/441 (33%)	222/458 (47%) 209/441 (46%)	39/458 (8%) 33/441 (7%)
AlbXVI	88	Transposase (363 aa) Transposase OrfA (88 aa)	*X. axonopodis* *Desulfovibrio vulgaris*	AF263433 AAC03166	64.8 bits (157) 61.0 bits (147)	2e-10 3e-09	27/45 (60%) 29/54 (53%)	40/45 (88%) 38/54 (69%)	- -

Table 10: Albicidin production assays with *X. axonopodis* pv. vesicatoria exconjugants harbouring different plasmids: analysis of growth inhibition of *E. coli* DH5αKT (susceptible to albicidin) and DH5αAlb^{r}KT (resistant to albicidin) in assays performed with different antibiotic combinations (no antibiotic, tetracycline only, kanamycin only and tetracycline+kanamycin).

		Combination of plasmids			
Bioassay medium containing	Tester strain	pUFR043 and pLAFR3	pUFR043 and pOp3-4/XALB2-3	PALB571 and pLAFR3	PALB571 and pOp3-4/XALB2-3
No antibiotic	DH5αKT	-	-	-	-
	DH5αAlb^{r}KT	-	-	-	-
Tetracycline	DH5αKT	-	-	-	+
	DH5αAlb^{r}KT	-	-	-	-
Kanamycin	DH5αKT	+	+	+	+
	DH5αAlb^{r}KT	+	+	+	+
Tetracycline+kanamycin	DH5αKT	+	+	+	+
	DH5αAlb^{r}KT	+	+	+	+

+: presence of a growth inhibition zone
All experiments were performed at least in duplicate with at least 2 exconjugants obtained from two independent triparental conjugations.

I hereby certify that this correspondence is being electronically filed in the United States Patent and Trademark Office on June 26, 2008.

Frank C. Eisenschenk, Ph.D., Patent Attorney

AMENDMENT UNDER 37 C.F.R. § 1.111
Patent Application
Docket No. UF-T398XC1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Hope A. Robinson
Art Unit	:	1652
Applicants	:	Monique Royer, Dean W. Gabriel, Roger Frutos, Philippe Rott
Serial No.	:	10/531,351
Filed	:	April 15, 2005
Conf. No.	:	6228
For	:	Biosynthetic Genes and Host Cells for the Synthesis of Polyketide Antibiotics and Method of Use

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

AMENDMENT UNDER 37 C.F.R. § 1.111

Sir:

In response to the Office Action dated March 26, 2008, please amend the above-identified patent application as follows:

In the Specification

Please substitute the following title of the invention on page 1:

~~COMPLETE BIOSYNTHETIC GENE SET FOR SYNTHESIS OF POLYKETIDE ANTIBIOTICS, INCLUDING THE ALBICIDIN FAMILY, RESISTANCE GENES, AND USES THEREOF~~

BIOSYNTHETIC GENES AND HOST CELLS FOR THE SYNTHESIS OF POLYKETIDE ANTIBIOTICS AND METHOD OF USE

Please substitute the following first paragraph on page 1:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is the U.S. national stage application of International Patent Application No. PCT/US2003/33142, filed October 17, 2003, which claims the benefit of U.S. Provisional patent application with Serial No. 60/419,463, filed October 18, 2002 ~~the disclosure~~ disclosures of which ~~is~~ are hereby incorporated by reference in ~~its~~ their entirety, including all nucleic acid sequences, amino acid sequences, chemical formulae, tables and figures.

Please insert the following paragraph on page 1, before paragraph [0001]:

The Sequence Listing for this application is labeled "seq-list-replace.txt" which was created on June 26, 2008 and is 323 KB. The entire contents of the sequence listing is incorporated herein by reference in its entirety.

Please substitute paragraphs [0015], [0016], [0017] and [0018] beginning on page 4 as follows:

Figure 5 is an illustration of the alignment of the primary sequences between the conserved motifs A4 and A5 of Alb NPRSs and PKS-4 in *Xanthomonas albilineans* with the corresponding sequences of GrsA (Phe) accession number: P14687 (SEQ ID NO: 132) and Blm NRPS-2 (β-Ala) accession number AF210249 (SEQ ID NO: 133); AlbI NRPS-1 (SEQ ID NO: 134); AlbI NRPS-3 (SEQ ID NO: 135); AlbIX NRPS-6 (SEQ ID NO: 136); AlbIX NRPS-7 (SEQ ID NO: 137); AlbIV

NRPS-5 (SEQ ID NO: 138); AlbVII PKS-4 (SEQ ID NO: 139); AlbI NRPS-2 (SEQ ID NO: 140).

Figure 6 shows Rho-independent transcription terminators identified in the intergenic regions of XALB1 and XALB3 clusters (SEQ ID NO: 141, XALB1 Strand + (29 bp downstream from the TGA stop codon of *albXVII*); SEQ ID NO: 142, XALB1 Strand + (400 bp downstream from the TAA stop codon of *albIV*); SEQ ID NOs: 143, 144 and 145, XALB1 Strand – (62 bp, 170 bp and 560 bp downstream from the TAG stop codon of *albXVI*); SEQ ID NOs: 146 and 147, XLAB3 Strand+).

Figure 7A shows sequences identified as a putative bidirectional promoter between albX and albXVII in XALB1 for transcriptional control of operons 3 and 4 (SEQ ID NOs: 148 and 149).

Figure 7B shows sequences identified as a putative unidirectional promoter upstream from *albXIX* for transcriptional control of operon 5 if *albXVIII* is not expressed (SEQ ID NOs: 152 and 153).

Please substitute paragraphs [0022] and [0023] beginning on page 5 as follows:

Figure 10A is an alignment of the conserved motifs in AT domains from RifA-1 (SEQ ID NO: 156), -2 (SEQ ID NO: 157), -3 (SEQ ID NO: 158), RifB-1 (SEQ ID NO: 159), RifE-1 (SEQ ID NO: 160) (Rifamycin PKSs, August *et al.*, 1998) and BlmVIII (Bleomycin PKS; Du *et al.*, 2000) (SEQ ID NO: 161).

Figure 10B is a comparison of AlbXIII (SEQ ID NO: 162), FenF (a malonyl-CoA transacylase located upstream from *mycA*, Duitman *et al.*, 1999) (SEQ ID NO: 163) and LipA (a lipase; Valdez *et al.*, 1999) (SEQ ID NO: 164).

Please substitute paragraph [00120] beginning on page 35 as follows:

albII potentially encodes a protein of 343 aa (AlbII) with a predicted size of 37.7 kDa. *albII* is 100% identical to the *xabC* cistron, previously described as encoding an *O*-methyltransferase downstream *xabB* (Huang *et al.*, 2000a). This conclusion is based on the similarity of XabC with a family of methyltransferases that utilize S-adenosyl-L-methionine (SAM) as a co-substrate for *O*-methylation including TcmO protein from *Streptomyces glaucescens* (Huang *et al.*, 2000a). AlbII contains three highly conserved motifs of SAM-dependent methyltransferases, including the motif I

involved in SAM binding (Figure 3). In the Figure, identical or similar amino acids (A=G; D=E; I=L=V) are shown in bold. Numbers indicate the position of the amino acid from the N-terminus of the protein. Abbreviations used in the Figure are: Sgl-TcmO (SEQ ID NOs: 55, 56 and 57) and Sgl-TcmN (SEQ ID NOs: 58, 59 and 60), multifunctional cyclase-hydratase-3-*O*-Mtase and tetracenomycin polyketide synthesis 8-*O*-Mtase of *Streptomyces glaucescens*, respectively (accession number: M80674); Smy-MdmC, midecamycin-*O*-Mtase of *Streptomyces mycarofaciens* (accession number: M93958) (SEQ ID NOs: 61, 62 and 63); Mxa-SafC, Saframycin *O*-Mtase of *Myxococcus xanthus* (accession number: U24657) (SEQ ID NO:s: 64, 65 and 66); Ser-EryG, erythromycin biosynthesis *O*-Mtase of *Saccharopolyspora erythraea* (accession number: S18533) (SEQ ID NOs: 67, 68 and 69); Spe-DauK, carminomycin 4-O-Mtase of *Streptomyces peucetius* (accession number: L13453) (SEQ ID NOs: 70, 71 and 72); Sal-DmpM, *O*-demethylpuromycin-*O*-Mtase of *Streptomyces alboniger* (accession number: M74560) (SEQ ID NOs: 73, 74 and 75); Shy-RapM, rapamycin *O*-Mtase of *Streptomyces hygroscopicus* (accession number: X86780) (SEQ ID NOs: 76, 77 and 78); Sav-AveD, avermectin B 5-*O*-Mtase of *Streptomyces avermitilis* (accession number: G5921167) (SEQ ID NOs: 79, 80 and 81), Sar-Cmet, mithramycin *C*-methyltransferase of *Streptomyces argillaceus* (accession number: AF077869) (SEQ ID NOs: 82, 83 and 84); AlbII, putative albicidin biosynthesis *C*-Methyltransferase of *Xanthomonas albilineans* (SEQ ID No. 27); identical to XabC, accession number: AF239749) (SEQ ID NOs: 85, 86 and 87).

Please substitute paragraph [00122] beginning on page 35 as follows:

albVI potentially encodes a protein of 286 aa (AlbVI) with a predicted size of 32.1 kDa similar to several hypothetical protein from *Mycobacterium tuberculosis* (Genbank accessions No. AAK46042, AAK48238, AAK44517, AAK46218) and from *S. coelicolor* (Genbank accession No. CAC03631). AlbVI is also similar to the tetracenomycine C synthesis protein (TcmP) of *Pasteurella multocida* (Table 4). Four highly conserved motifs in TcmP and other *O*-methyltransferases are also present in AlbVI (Figure 4), suggesting that AlbVI is an *O*-methyltransferase. In the Figure, identical or similar aa (A=G; D=E; I=L=V; K=R) are shown in bold. Numbers indicate the position of aa from the N-terminus of the protein. Abbreviations used in the Figure are: Sgl-tcmP, tetracenomycin C synthesis protein of *Streptomyces glaucescens* (accession number: C47127) (SEQ ID NOs: 88, 89,

90 and 91); Sme-PKS, putative polyketide synthase of *Sinorhizobium meliloti* (accession number: AAK65734) (SEQ ID NOs: 92, 93, 94 and 95); Pmu-tcmP: tetracenomycin C synthesis protein of *Pasteurella multocida* (accession number: AAK03406) (SEQ ID NOs: 96, 97, 98 and 99); Mtu-Omt: putative *O*-methyltransferase of *Mycobacterium tuberculosis* (accession number: AAK45444) (SEQ ID NOs: 100, 101, 102 and 103); Mlo-Hp: hypothetical protein containing similarity to *O*-methyltransferase of *Mesorhizobium loti* (accession number: BAB50127) (SEQ ID NOs: 104, 105, 106 and 107); ~~Mtu-Hp1~~Mtu-Hp: hypothetical protein of *Mycobacterium tuberculosis* (accession number: AAK46042) (SEQ ID NOs: 108, 109, 110 and 111); Mtu-Hp2: hypothetical protein of *Mycobacterium tuberculosis* (accession number: AAK48238) (SEQ ID NOs: 112, 113, 114 and 115); Mtu-Hp3: hypothetical protein of *Mycobacterium tuberculosis* (accession number: AAK44517) (SEQ ID NOs: 116, 117, 118 and 119); Mtu-Hp4: hypothetical protein of *Mycobacterium tuberculosis* (accession number: AAK46218) (SEQ ID NOs: 120, 121, 122 and 123); Sco-Hp: hypothetical protein of *Streptomyces coelicolor* (accession number: CAC03631) (SEQ ID NOs: 124, 125, 126 and 127); AlbVI, putative albicidin biosynthesis *O*-Methyltransferase of *Xanthomonas albilineans* (this study) (SEQ ID NOs: 128, 129, 130 and 131).

Please replace pages 1-93 (Sequence Listing) submitted on April 25, 2005 in the subject application with new pages 1-180 submitted herewith.

In the Claims

1-61 (Canceled).

62 (new). A transformed host cell that comprises one or more genetic construct that comprises SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3, wherein said transformed host cell produces albicidin.

63 (new). The transformed host cell of claim 51, wherein said one or more genetic construct contains a combination of SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

64 (new). The transformed host cell of claim 51, wherein said host cell has been transformed with a first genetic construct comprising SEQ ID NO: 1, a second genetic construct comprising SEQ ID NO: 2 and a third genetic construct comprising SEQ ID NO: 3.

65 (new). A transformed host cell comprising a one or more polynucleotides encoding a biosynthetic pathway for albicidin production, said onc or more polynucleotides encoding SEQ ID NO: 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, and 47 and wherein said host cell produces albicidin.

66 (new). A combination of isolated polynucleotides that encode SEQ ID NO: 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, and 47.

67 (new). The combination of isolated polynucleotides according to claim 66, wherein said combination of polynucleotides comprises SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

68 (new). The combination of isolated polynucleotides according to claim 66, wherein said polynucleotides that encode SEQ ID NO: 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39,

40, 41, 42, 43, 44, 45, 46, and 47 are SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, and 25.

69 (new-withdrawn). A method of making an antibiotic comprising the culturing of a transformed host cell according to claim 65 under conditions that allow for the production of said antibiotic.

70 (new-withdrawn). The method according to claim 69, further comprising the isolation of said antibiotic.

Remarks

Claims 51-61 are pending in the subject application. Applicants acknowledge that claims 60 and 61 have been withdrawn from further consideration as being drawn to a non-elected invention. By this Amendment, Applicants have canceled claims 51-61 and added new claims 62-70. Support for the new claims can be found throughout the subject specification (see, for example, paragraph 45) and in the claims as originally filed. Entry and consideration of the new claims presented herein is respectfully requested. Accordingly, claims 62-70 are currently before the Examiner with claims 69 and 70 standing withdrawn from consideration. Favorable consideration of the pending claims is respectfully requested.

The specification is objected to because of the following informalities: the title of the invention is not descriptive. Applicants have adopted the Examiner's suggestion and changed the title of the invention to: "Biosynthetic Genes and Host Cells for the Synthesis of Polyketide Antibiotics and Method of Use." Accordingly, reconsideration and withdrawal of the objection is respectfully requested.

The specification is objected to because of the following informalities: Figures 3-7 and 10 disclose sequences, however, the Brief Description of the Drawing does not report the corresponding SEQ ID NO. Applicants have revised the Examples to provide SEQ ID NOs. for the pertinent sequences found in the figures and respectfully submit that this issue is now moot. In addition, a replacement sequence listing has been provided and the necessary paragraph added to the subject specification. I hereby certify that no new material is being added by this submission. Accordingly, reconsideration and withdrawal of the objections is respectfully requested.

Claims 52 and 55 are objected to because of the following informalities: claim 52 depends from a rejected base claim and claim 55(j) is suggested by the Examiner to be amended to read, "isolated polynucleotide sequence." Applicants thank the Examiner for her careful review of the claims and have rewritten the new claims in a fashion that renders this issue moot. Accordingly, reconsideration and withdrawal of the objections is respectfully requested.

Claims 54-59 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the

claimed invention. The Office Action argues that the as-filed specification fails to provide adequate written description for the fragments of SEQ ID NOs: 1, 2 and 3 previously recited within the claims. Applicants respectfully disagree and traverse.

Previously pending claim 54 recites "a combination of nucleotide fragments of SEQ ID NOS:1-3." The specification identifies these sequences as DNA sequences as physically separate coding regions of *X. albilineans* DNA that provide a biosynthetic pathway for the production of albicidin. These sequences represent the genetic clusters XALB1, XALB2, and XALB3 which contain the 22 genes necessary for albicidin biosynthesis. Applicants have provided SEQ ID NOS:1-3 (nucleotide sequences for XALB1, XALB2, and XALB3) and have also elucidated the functional components of SEQ ID NOS:1-3 by reading frame analysis (Example 5). In addition to examining the functions of each individual gene, Applicants used the claimed genetic sequences to create genetic constructs for the ultimate purpose of albicidin biosynthesis (page 48 of the specification). Applicants also maintain that the inclusion of SEQ ID NOs:4-47 and/or SEQ ID NOs: 4-25 specifically identify the individual genetic elements of SEQ ID NOS:1-3 necessary for albicidin biosynthesis and exemplify Applicants' possession of polynucleotide fragments derived from SEQ ID NOs: 1, 2 and 3. Accordingly, withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claims 53 and 55 are rejected under 35 U.S.C. § 112, second paragraph, as failing to set forth the subject matter, which applicant(s) regard as their invention. With respect to the issue noted in claims 53 and 55, it is respectfully submitted that the new claims have rendered these issues moot and reconsideration and withdrawal of the rejection is respectfully requested.

Claims 51 and 54-59 are rejected under 35 U.S.C. § 102(b) as being anticipated by Huang *et al.* (2000). The Office Action argues that the disclosure of Huang *et al.* anticipates the claimed invention and notes that Huang *et al.* teach a gene (*xabA*) for albicidin biosynthesis and teach the use of a host cell such as *E.coli* for production of albicidin. Applicants respectfully disagree with the Examiner's finding of anticipation. As the Patent Office is aware, a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). Claim 51 requires a "genetic construct that comprises SEQ

ID NO: 1, SEQ ID NO: 2, **and** SEQ ID NO: 3 (emphasis added). Huang *et al.* fail to teach these additional sequences; thus, anticipation of the claimed invention cannot exist. Applicants also disagree with the Examiner's determination that Huang *et al.* teach the use of *E. coli* for albicidin production. Huang *et al.* specifically note the inability of *E. coli* to produce albicidin upon addition of clones spanning the putative main albicidin biosynthesis region (page 198, col. 2, paragraph 3). Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 102(b) is respectfully requested.

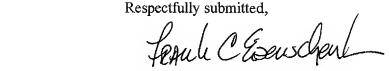
It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position. Applicants expressly reserve the right to pursue the invention(s) disclosed in the subject application, including any subject matter canceled or not pursued during prosecution of the subject application, in a related application.

In view of the foregoing remarks and amendments to the claims, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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FCE/sl
Attachment: New pages 1-180 (Sequence Listing) of the subject specification

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,510,852

APPLICATION NO.: 10/531,351

DATED : March 31, 2009

INVENTORS : Monique Royer, Dean W. Gabriel, Roger Frutos, Philippe Rott

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 4,
Line 23, "P14687 SEQ ID NO: 132" should read --P14687 (SEQ ID NO: 132)--.
Line 56, "RitE-1" should read --RifE-1--.

Column 10,
Line 60, "Vols. I, II, and II" should read --Vols. I, II, and III--.

Column 16,
Line 19, "Proc. Natl. Acad. Sci. U.S." should read --Proc. Natl. Acad. Sci. U.S.A.--.
Line 23, "Proc. Natl. Acad. Sci. U.S." should read --Proc. Natl. Acad. Sci. U.S.A.--.

Column 22,
Line 21, "protecting a plant; against" should read --protecting a plant against--.
Line 40, "kanamnycin" should read --kanamycin--.

Column 26,
Line 31, "(5'tgeccacaggccgtcgagt3')" should read --(5'tgcccacaggccgtcgagt3')--.

Column 27,
Line 4, "SEQ D No: 6" should read --SEQ ID No: 6--.
Line 51, "J-ketoacyl synthase" should read --β-ketoacyl synthase--.

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It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 29,
Line 22, "albXV" should read --albXI--.
Line 60, "motifI involved" should read --motif I involved--.

Column 31,
Line 15, "AlbXVI" should read --AlbXVII--.
Line 20, "albXVII" should read --albXVIII--.
Line 21, "AlbXIII" should read --AlbXVIII--.
Lines 35-36, "(5'cgttgaggatgcagcgctcg31')" should read --(5'cgttgaggatgcagcgctcg3')--.

Column 34,
Line 7, "from *Comamnonas*" should read --from *Comamonas*--.

Column 36,
Line 9, "BLm (&Ala)" should read --Blm (β-Ala)--.
Line 27, "confined" should read --confirmed--.
Line 58, "from albX" should read --from albXIX--

Column 40,
Lines 63-64, "and XhoSalXaHT1PGF 5'cgatatcgccttcctcgagggtategataagc3'" should read --and XhoSalXaHTPGF 5'cgatatcgccttcctcgagggtatcgataagc3'--.

Column 41,
Line 14, "pUFR043-pOp34/XALB2-3" should read --pUFR043-pOp3-4/XALB2-3--.
Line 15, "pAlb571-pOp3A4XALB2-3" should read --pAlb571-pOp3-4/XALB2-3--.

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It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 42,
Line 29, "Because albXVII" should read --Because albXVIII--.
Line 46, "confining" should read --confirming--.

Column 43,
Line 51, "stain Xa13" should read --strain Xa13--.
Line 64, "and albIV)" should read --and albIX)--.

Column 45,
Line 47, "PKS4 module" should read --PKS-4 module--.

Column 47,
Line 58, "AbVII HBCL" should read --AlbVII HBCL--.

Column 49,
Line 14, "identical to albX)" should read --identical to albXXI)--.

Column 51,
Table 1, row "pBC/f", "2.5 kb Kpn I-EdoR I" should read --2.5 kb Kpn I-EcoR I--.

Column 53,
Table 1, row "pBKS/XALB3XhoI", "pBKS/XALB3 with a KhoI site" should read --pBKS/XALB3 with a XhoI site--.

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INVENTORS : Monique Royer, Dean W. Gabriel, Roger Frutos, Philippe Rott

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 60,
Table 4, row "AlbXIII", Columns "Identities", "Positives", "Gaps",
"43/156 (28%)" 56/156 44/156
should read --43/156 56/156 44/156 (28%)--.

Column 66,
Table 10, row "Tetracycline",
"DH5αKT DHSαAlb'KT" should read --DH5αKT DH5αAlb'KT--.

Column 264,
Line 26, "36, 37, 39, 39, 40" should read --36, 37, 38, 39, 40--.
Lines 30-31, "36, 37, 39, 39, 40" should read --36, 37, 38, 39, 40--.

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